

The University of Chicago

STUDIES ON THE GROWTH FAC-
TORS FOR PATHOGENIC
BACTERIA

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

1937

By
RAYMOND DANIEL FINKLE

Private Edition, Distributed by
THE UNIVERSITY OF CHICAGO LIBRARIES
CHICAGO, ILLINOIS

1941

The University of Chicago

STUDIES ON THE GROWTH FAC-
TORS FOR PATHOGENIC
BACTERIA

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

1937

By
RAYMOND DANIEL FINKLE

Private Edition, Distributed by
THE UNIVERSITY OF CHICAGO LIBRARIES
CHICAGO, ILLINOIS

1941



ACKNOWLEDGMENTS

The writer is greatly indebted to Dr. Felix Saunders for his invaluable help in the preparation of this study. Many thanks are due to Dr. S. A. Koser for the assays and for his co-operation and to Mr. Albert Dorfman, Dr. Leon Sternfeld, and Mr. William Wermuth for their help in gathering the analytical data.

This work has been aided by a grant from the Committee on Scientific Research of the American Medical Association. It is a pleasure to be able to thank the Committee for the generous support.



Digitized by the Internet Archive
in 2026

https://archive.org/details/IA41533910_0061

INTRODUCTION

Many bacteria which are capable of growth on meat infusions, peptone, broth, etc. grow poorly, if at all, on a mixture of amino acids, dextrose, and salts. This group of so-called "fastidious bacteria" includes most of the pathogenic organisms. It can be demonstrated that the addition of a few micrograms or less of a plant or animal tissue concentrate to a "synthetic" medium (a medium whose ingredients are of known chemical constitution and of high purity) modifies the medium to the extent of permitting luxuriant growth. This phenomenon has led to the postulation of vitamin-like substances or growth factors as necessary parts of bacterial nutrition.

The very extensive literature on this subject has been the subject of reviews by Peskett (13), Burrows (2), and Knight (7). Much of the older work was done using peptone and other media containing substances of unknown composition and questionable purity. It can be shown that peptones of highest purity contain substances which are soluble in alcohol and which are capable of acting as bacterial growth stimulants. Sahyan and his co-workers (16) have used this type of preparation routinely as a source of the growth stimulant for the colon bacillus. The use of protein acid hydrolysates as an amino acid base is another source of uncertainty in these studies and is generally being abandoned in favor of the more exact, though expensive, synthetic medium containing purified amino acids.

Pappenheimer (14), working with a factor for Clostridium sporogenes, obtained a preparation from mare pregnancy urine which was active in a concentration of 0.04 microgram/cc. The formula of $C_{10}H_{12}O_4$ was assigned to the compound which appeared to be an optically inactive unsaturated acid. Further purification was unsuccessful; the benzoyl and hydrazide derivatives were oils while the barium, zinc, and lead salts were hygroscopic and formed resins on drying. He attempted to obtain similar preparations from yeast extracts but was unsuccessful. It is possible that a different compound is present in yeast which has a similar biological

activity.

Knight worked on a factor for Staphylococcus aureus and obtained a preparation from marmite, a commercial yeast concentrate, which was active in concentration of 0.03 micrograms/cc. He shared with Hughes (5) the idea that the Staphylococcus factor is a nitrogenous base. Hughes based this contention on the completeness of the precipitation of the activity with phosphotungstic acid. Knight, on the other hand, finds this precipitation to be incomplete and considerable activity is lost, presumably by adsorption onto the inert precipitates. The presence of 5.6% nitrogen in an active distillate and the extractability of the activity by butyl alcohol from alkaline aqueous solutions support his views.

Richardson (15) in studying the anaerobic growth requirements of Staphylococcus aureus demonstrated the necessity of purines or pyrimidines for this phase of the organism's growth. Uracil was found to be the most active compound of the group and its activity could be demonstrated at a concentration of 0.05 micrograms/cc.

Mueller (12) isolated a growth stimulant for Corynebacterium diphtheriae from cow's urine which he has identified with synthetic pimelic acid. Concentrations of 0.005-0.10 micrograms/cc. in a casein hydrolysate medium containing a small amount of water soluble factor from mammalian liver caused a three to five-fold increase in growth as measured by the bacterial nitrogen. Azelaic acid was also found in the urine but was inactive.

The adsorption of the active material by charcoal had been observed by many workers (11, 16, 6). It was shown by Koser and Saunders (9) that the material adsorbed from veal infusions could be eluted from the charcoal by hot alcoholic solutions. Aqueous solutions containing this eluate were added to synthetic media and were found to support the growth of representatives of the typhoid and dysentery groups, the diphtheria bacillus, Staphylococcus albus, and Brucella abortus. Transplantations through twenty to thirty transfers in synthetic media containing 1.2 micrograms/cc. were carried out with good growth throughout. Under the conditions studied no growth was obtained with several strains of scarlet fever and septic sore throat streptococci, Streptococcus lactis and Pasteurella avicida. Using the same technique of adsorption and elution a study of the distribution of the active ma-

terial was made (10). Preparations from some twenty-five sources of plant and animal origin were made and almost all were found to be active. Calf spleen and to a slightly lesser extent calf liver and bakers' yeast were found to be particularly rich in this factor and were used as starting materials for subsequent work (17).

From the results of these experiments it appeared that a single growth factor was widely distributed in living tissue in amounts which varied with the source and was essential to the growth of a large number of the pathogenic organisms studied. While differences in the responses of various organisms to the added material were apparent, these differences appeared to be of a quantitative rather than a qualitative nature. It was possible to arrange the organisms in a linear order based on the growth response to a particular extract and this order was retained regardless of the source of the extract. Some of the organisms which did not grow with preparations from veal infusions, such as Streptococcus scarlatinae, Streptococcus lactis, and Pasteurella avicida, responded to the extracts from calf spleen and traces of growth were observed with liver and yeast preparations. No differences in stability or solubility were observed and the idea of a single essential growth factor for many pathogenic organisms was adopted as a reasonable working hypothesis. The testing of this idea, the purification, and the elucidation of the chemical properties of the essential factor forms the subject matter of this study.

METHODS AND PROCEDURES

Culture Medium

The synthetic medium used had the composition shown in Table 1. The medium was tubed in 5 cc. quantities and sterilized by autoclaving at twelve pounds pressure for twenty minutes. For the inorganic salt solutions used in the controls the asparagine, cystine, tryptophane, and dextrose were omitted.

TABLE 1

Na_2HPO_4	1.40 gm.
KH_2PO_4	1.00
NaCl	2.00
MgSO_4	0.10
Asparagine	3.00
Cystine	0.10
Tryptophane	0.20
Dextrose	2.00
Water (Redistilled)	1000 cc.

Organisms

The organisms used for assay were a laboratory stock culture strain of Staphylococcus albus, Corynebacterium diphtheria (Park 8), Shigella dysenteriae (Shigella 76), and Brucella abortus (456). These organisms were chosen for differences in their requirements rather than similarities; the first two organisms were much more responsive to the growth factor than were the last two.

Method of Assay

The solutions to be tested were autoclaved at twelve pounds for twenty minutes and 0.10 cc. of each and of decimal serial dilutions was pipetted into a series of tubes each containing 5 cc. of synthetic medium. A small inoculum of the test organism was transferred to a tube containing 5 cc. of inorganic salt solution and the tube was shaken to disperse the inoculum. One-tenth cc. of this suspension which had no evident turbidity was pipetted into the previously prepared tubes and into the control tubes. The tubes were then incubated at 37°C. and readings were made after one, two, and four days. The assays were checked after ten days to note any delayed growth that might have occurred. The amount of growth was judged by the turbidity observed on shaking the tubes. Full growth (i.e., comparable to that obtained in a veal infusion broth) was indicated by ++++; and lesser amounts of growth by +++, ++, +, and +^s; and barely observable turbidity by (?).

Control tubes of (1) the organisms in inorganic salt solu-

tion and growth factor solution, (2) the organisms in the synthetic medium alone, and (3) the organisms in veal infusion broth were used. Growth in control tube (1) indicated that the tested solution contained sufficient nutrient material to support growth and was acting as more than an accessory factor. Only weak growth (+ or +^s) was occasionally observed in the first (undiluted) tube with concentrated tested solutions only. Growth in control tube (2) would indicate that the inoculum was too heavy or that the organism had been "trained" to grow without the accessory factors (6). Although growth was occasionally observable in these controls, the latter interpretation was not possible since repetition of the assay would invariably show a lack of growth in this control tube. Growth in control tube (3) was used for comparative purposes and to assure the viability of the organisms.

This type of assay is neither exact nor sensitive but there is no present method which has a very high order of accuracy. All other methods are tedious and would not give an essentially better picture of the presence or absence of growth factor. The plate count method is very time-consuming. Furthermore this method measures only the number of bacteria which are viable at any one time and does not give a measure of the total conversion of energy and foodstuffs to bacterial protoplasm which had taken place over the entire period of time. The method of bacterial nitrogen determination is perhaps the most accurate one available to date and affords a measure of the total activity over a certain period. It has the disadvantage of necessitating the stopping of growth at the time of analysis and introduces an arbitrary factor. For our purposes the method used was quite satisfactory. It is by far the simplest and least expensive method available. Its accuracy using decimal serial dilutions is ample for the demonstration of the delicate phenomenon at hand and for the routine assays. Improvement of the method by the use of serial dilutions in steps of two to five is always available and on occasion has been used.

Determination of Solid Content

The total solid content of a solution was determined by drying a measured amount of the solution (usually one cc.) at 110°C. for two hours. The residue was weighed and the weight expressed in per cent was called the total solid content. The residue was then slowly heated in a flame until all charring had

ceased and then heated in the full flame of the bunsen burner for fifteen minutes. The weight of the residue expressed in per cent was called the ash content. This ash content was deducted from the total solid content and the difference called the organic solid content.

Determination of Nitrogen

Total nitrogen content of these solutions on properly diluted samples (to give 0.2-0.8 mgm./100 cc. for the Nesslerization) was determined by the Koch-McMeekin method (8). These values were expressed as per cent of the organic solid content. Amino nitrogen content of the solutions was determined by the Van Slyke manometric method (18) (using a constant time interval of four and one-half minutes) and the amino nitrogen values were expressed as per cent of the total nitrogen content.

Elementary Analysis

One-tenth to one cc. of solution were placed in a pyrex test-tube and dried at 110°C . for one hour. A small pellet of cleaned sodium metal was dropped into the tube and heat was cautiously applied until the vigorous reaction began. The flames were withdrawn until the reaction subsided. Then the tube was strongly heated for one minute. After the tube had cooled, alcohol was poured into the tube to decompose the sodium. The contents of the tube were extracted three times with water. The extracts were combined, concentrated to a small volume, and filtered. The examination for the elements was made in the regular manner.

Preparation of the Active Solutions

Five kg. of fresh tissue (spleen or liver) were ground and stirred into 50 liters of water and the suspension was placed in the icebox overnight. The suspension was heated to 80°C . with constant stirring. Heating was continued until the boiling point was reached and this temperature was maintained for fifteen minutes. The suspension was then filtered hot through cheesecloth and then through paper. Fifty grams each of washed fullers' earth and supercel were added to the filtrate and the suspension was

stirred for fifteen minutes and filtered. Five grams of charcoal (Carboraffin) were stirred into the filtrate (pH 6.8-7.4) every five minutes until a total of twenty-five grams of charcoal had been added. The charcoal was filtered off under suction. The wet charcoal from eight to ten such preparations (50 kg. of fresh tissue) was placed in a large continuous extractor and extracted with 90% alcohol for two weeks. The alcohol was changed after the first twenty-four hours and after that every two to five days depending on how concentrated and deeply colored the solution became. The alcoholic solutions were combined, filtered, and evaporated to dryness under reduced pressure. The residue was taken up in 100 cc. of water and any insoluble material was filtered off and the solution was set aside in the icebox at 3-5°C. The solution had a solid contents of 1-5% of which one-tenth was ash and had a pH of 6.7-7.0. The material was active when used in the bacterial medium in a concentration of 0.5-50 micrograms/cc. Elementary analysis of these solutions show the absence of sulfur and phosphorus, and the presence of a trace of halogens. Nitrogen is present in abundance.

ELUTION EXPERIMENTS

It was early noted that the elution was slow and that organic material and active material were still being eluted at the end of the two week period. It was considered important to know more about the rate of elution and a series of experiments using water, 90% alcohol, and 25% alcohol were performed in which the elution was discontinued periodically and the elutate of each period examined separately. The results are summarized in Tables 2, 3, and 4.

The activity obtained using water compares favorably with that obtained using 90% alcohol or 25% alcohol. The differences seem to lie in the amount of extraneous material obtained rather than in the active material eluted. The activity is apparently held very tightly by the charcoal while much of the elutable inert material is less firmly held with the result that the activity is eluted at a very slow, steady rate while the inert material comes off relatively more rapidly. Consequently, the second, third, and fourth elutates are as active as the first and, in some cases,

TABLE 2

ELUTION WITH WATER FOLLOWED BY 90% ALCOHOL

Elut- ate No.	Time in Days	Total Wgt. in Gms.	Total Wgt. in Gms.	Per cent Extracted Wgt. x 100 Total Wgt.	Rate of Extraction (gms./day)	Per cent of Nitrogen	Activity (Minimum Effective Amount) in Micrograms/cc.			
							C. diph.	Staph. albus	Shigella dysen- teriae	Brucella abortus
1	1 1/2	2.01	2.01	48	21	1.35	2.7	27	270	270 ^b
2	5 1/2	1.06	3.07	73	31	0.63	1.1	11	106	i ^b
3	14	1.18	4.20	100	43	0.30	1.1	11	113	i
4 ^a	3	4.11	8.33	...	85	1.37	4.1	41	411	i
5	11	0.97	9.30	...	95	0.088	9.7	97 ^b	i ^c	i
6	10	0.45	9.75	...	100	0.045	45.0 ^b	450	i	i

^aWater replaced by 90% alcohol after elutate no. 3. Elutates nos. 4, 5, and 6 were made with 90% alcohol.

^bGrowth was observed in the next dilution to the one taken into account in determining the activity. This growth was observed on the 10th day and, therefore, was not counted.

^cThe symbol "i" indicates that the solution was inactive in the concentrations studied.

TABLE 3

ELUTION WITH 90% ALCOHOL

Elut- ate No.	Time in Days	Total Time in Days	Weight in Grams	Total Weight in Grams	Per cent Extracted	Rate of Extraction	Per cent of Nitrogen	Activity in micrograms/cc.			
								C. diph.	Staph.	Shiga.	Brucella
1	1 1/2	1 1/2	7.98	7.98	80	5.32	19.6	11	11	1,060	106
2	4 1/2	6	0.85	8.83	88	0.17	17.4	17	170	i ^a	170
3	7	13	0.61	9.94	94	0.087	15.3	12	120	i	i ^b
4	15	28	0.64	10.08	100	0.045	15.1	11	114	i	i

^aThe symbol "i" indicates that the solution was inactive in the concentrations studied.

^bGrowth was observed in the next dilution to the one taken into account in determining the activity. This growth was observed on the 10th day and, therefore, was not counted.

TABLE 4
ELUTION WITH 25% ALCOHOL

Elut- ate No.	Time in Days	Total Time in Days	Weight in Grams	Total Weight in Grams	Per cent Extracted	Rate of Extraction	Per cent of Nitrogen	Activity in micrograms/cc.			
								C. diph.	Staph.	Shiga.	Brucella
1	1 1/2	1 1/2	17.00	17.00	68	11.30	19.2	35.0	35	350	3,500 ^a
2	3 1/2	5	4.95	21.95	88	1.65	22.1	10.0	100	1,000	1,000
3	10	15	1.37	23.32	93	0.14	16.5	2.7	27	270	270
4	10	25	0.86	24.28	98	0.086	15.1	17.0 ^a	17	1 ^b	170
5	10	35	0.52	24.80	100	0.052	14.6	100.0 ^a	100 ^a	1	1

^aGrowth was observed in the next dilution to the one taken into account in determining the activity. This growth was observed on the 10th day and, therefore, was not counted.

^bThe symbol "1" indicates that the solution was inactive in the concentrations studied.

on a total weight basis, more so. The rates of elution with these three solvents appear to have approached a constant value of 50-100 mgm./day with the activity becoming progressively weaker. The continuation of the elution beyond this point was futile since the yield of material with any activity was low and the active material was being diluted with inert material which was bound to the adsorbent even more tightly than it was itself. As might be expected, most of the material (50-80%) was eluted in the first thirty-six hours. While in some cases this elutate is not as active as some of the material obtained later, still it contained most of the total activity and was a valuable source of the active substance. The nitrogen values in all cases started at about 20% and gradually decreased to about 15%. It is interesting to note that the nitrogen content of the solution before adsorption was 10-12%. The activity appears, therefore, to have been adsorbed with the nitrogenous compounds and to have been eluted with them. The idea that the compound itself contains nitrogen is suggested.

The activity of the solutions toward the various organisms show several interesting features. Certain differences in the behavior of the several organisms are worthy of note. (1) The results in Table 2 (water elution) show Shigella dysenteriae to be more responsive than Brucella abortus, but in Table 3 (90% alcohol elution) the order is inverted. (2) In Table 4 the order appears to invert itself, Brucella abortus being less responsive than Shigella dysenteriae to elution no. 1 and more responsive to elution no. 4. It seems that there are certain differences in extractability of the factors for Brucella abortus and Shigella dysenteriae which had not appeared in previous studies. On the other hand, the regular order of response by Corynebacterium diphtheriae, Staphylococcus albus, and Shigella dysenteriae holds in these cases. Corynebacterium diphtheriae is somewhat less than ten times as responsive as the Staphylococcus albus and about one hundred times as responsive as the Shigella dysenteriae in almost all the cases. These experiments point to the idea that the problem is more complex than that which would fit a "Unitary Theory" of one accessory factor (and only one accessory factor) for a large number of organisms.

It had been previously shown that elution of the activity by cold aqueous solutions of N/100 hydrochloric acid or N/100 sodium hydroxide or cold water were ineffective (9). These experi-

ments were repeated and similar results were obtained. Elution with cold (20-25°C.), dilute aqueous solutions of ammonia and acetic acid were tried. The charcoal was suspended in the solutions and the flasks were shaken for an hour. The suspensions were filtered and the charcoal extracted again with the solvents. After filtration the filtrates were combined, evaporated to dryness in order to remove the volatile acetic acid and ammonia, and taken up in a volume of water equal to one-fiftieth of the volume of solvent used. The pale yellow solutions had a total solid content of 1-2% and were able to induce growth for Corynebacterium diphtheriae and Staphylococcus albus in tubes containing only the lowest dilutions (1-50) of these test solutions. Equally weak though active solutions having smaller solid contents (0.2-0.5%) were obtained by repeating the process using distilled water as the eluting agent. The growth induced by these solutions was delayed and weak. Since the three solvents were equally ineffective in eluting the active material from the charcoal, no evidence was obtained for the existence of acidic or basic groups in the active molecule.

Further experimentation was carried out with dilute aqueous solutions of ammonia and acetic acid using the continuous extraction apparatus and boiling solvents. By this method, solutions were obtained which showed differences between acid and base treatment. The ammonia treatment produced a solution which was definitely weaker than alcohol or water controls and the solutions prepared from acetic acid elutates were as strong as, or slightly stronger than, these controls. From this it would seem that basic properties are indicated. There are, however, several complications which make this conclusion questionable. With forty hours of extraction the solutions from both solvents became very concentrated (15-20 gm. of water-soluble material and an equal amount of water-insoluble material) and very dark. One-tenth cc. of such a solution contained 15-20 mgm. of solids and was added to 5 cc. of synthetic medium which contained 26 mgm. of organic solids in all. One hesitates to consider results with such preparations in a study of accessory factors. Some of the solutions derived from the acetic acid treatment caused precipitates to form when as little as 0.01 cc. was added to 5 cc. of synthetic medium. Their potency had to be determined by streaking on veal infusion agar slants. Because of these considerations, hot elution with ammo-

nia or acetic acid solutions offered but little help in the preparation of active fractions or in deciding questions of chemical properties.

CHEMICAL STUDIES

Effect of Heat and Oxidants

1. Ten cc. of an active solution were evaporated to dryness in a platinum crucible. The dried material was ashed and the crucible was finally heated in the full flame of the bunsen burner for ten minutes. The crucible was cooled to room temperature and the ash (15 mgm.) was taken up in 10 cc. of water. Assay of this solution proved it to be inactive.

2. Because of the possibility of loss by volatilization in the preceding experiment, the conditions of the ashing were modified. Ten cc. of an active solution were evaporated to dryness in a porcelain boat. The boat was introduced into a combustion tube which was connected directly (all-glass) to a series of three small water traps each containing 5 cc. of water. The combustion tube was set in a furnace and the flames were slowly raised to allow for as much distillation into the water traps as possible. The flames were finally raised under the boat and the combustion was continued for thirty minutes and the tube was allowed to cool. Considerable tarry material was still present in the boat and a small amount of oily material had collected in the cooler end of the tube. The first water trap had a distinct yellow tint. The contents of the boat and of the tube were separately washed with water and with 1% hydrochloric acid. These solutions were evaporated to dryness, the residues were taken up in 5 cc. of water and the solutions were filtered. The contents and the washings of the traps were combined, evaporated to dryness, and the residues were taken up in 5 cc. of water. These three solutions were tested for activity both separately and combined. No activity was found to have resisted the treatment. These experiments were repeated using less prolonged heating and with less direct heating of the boat. It was found that in all cases where considerable carbonization of the organic material was attained the activity was destroyed.

3. The active material was subjected to heat-treatment and

no loss of activity was found after (a) autoclaving at fifteen pounds pressure (120°C.) for twenty minutes, (b) heating at 100°C. for three hours either as the dry powder or (c) in aqueous solution. The resistance to aqueous solutions of acids and alkalis is high. The results of treatments with acid and alkali will be discussed later.

4. The active material is resistant to oxidizing agents.

(a) Vigorous aeration at room temperature for twenty hours did not destroy the activity.

(b) The dried residue from 10 cc. of an active solution was dissolved in 10 cc. of concentrated sulfuric acid and was kept at room temperature for forty-eight hours. The solution became very dark-brown and after dilution with water a small amount of black precipitate was filtered off. An excess of lead carbonate was added to the filtrate and dissolved lead was removed with hydrogen sulfide. The solution was evaporated to dryness and the residue was taken up in 10 cc. of water. The results of such treatment are shown in Table 5. A reduction in ac-

TABLE 5

EFFECT OF CONCENTRATED SULFURIC ACID AT ROOM TEMPERATURE

	Amount of Extract in cc.	Staph. albus				C. Diphtheria				Shiga				Brucella			
		Days				Days				Days				Days			
		1	2	4	10	1	2	4	10	1	2	4	10	1	2	4	10
Before H ₂ SO ₄ Treat- ment	0.1	+ ^s	++	+++	+++	++	+++	+++	+++	+	+	+	+	+	++	++	++
	.01	+ ^s	+	++	+++	+	++	+++	+++	.	.	.	.	.	..	..	+
	.001	.	?	?	+	..	+	++	+++	.	.	.	.	.	..	..	..
	none	.	..	...	...	..	...	...	...	.	.	.	.	.	..	..	..
After H ₂ SO ₄ Treat- ment	0.1	+	+++	+++	+++	+	+	+++	+++	.	.	.	.	.	..	..	+
	.01	.	...	+	+	..	...	+	++	.	.	.	.	.	..	..	+
	.001	.	...	...	...	..	...	?	+ ^s	.	.	.	.	.	..	..	..
	none	.	...	...	...	..	...	...	...	.	.	.	.	.	..	..	..

tivity towards each organism was observed. The complete lack of growth of Shigella dysenteriae and the greatly retarded and weakened growth of Brucella abortus are suggestive of differences in the stability of two factors rather than one. The results can be explained, however, on the basis of a single factor whose concentration was reduced to a point below the minimum requirement for Shigella dysenteriae and just above the minimum requirement of Brucella abortus. The weakened responses with Staphylococcus albus and Corynebacterium diphtheriae are in agreement with this explanation. The results were similar when the treatment was carried out at 80°C. Heating the solution to fuming for thirty to sixty seconds resulted in the complete destruction of the activity.

(c) The activity can be destroyed by the use of hydrogen peroxide. Ten cc. of 30% hydrogen peroxide was added to 10 cc. of an active solution and the solution was evaporated to dryness on the steam bath. The residue was dissolved in 10 cc. of 30 hydrogen peroxide and the solution was re-evaporated. This process was repeated once more and the final faintly yellow residue was taken up in 10 cc. of water. This solution was found to be inactive. Treatment of the active solutions with 30% hydrogen peroxide for a fewer number of times resulted in partial loss of activity with Staphylococcus albus and Corynebacterium diphtheriae and complete loss of activity with Shigella dysenteriae and Brucella abortus. Treatment with 3% hydrogen peroxide for one to two hours had no perceptible effect on the activity.

(d) The effects of bromine were similar. Liquid bromine added to the dry preparation resulted in complete inactivation of the material, while saturated aqueous solutions of bromine were ineffective in causing any change in the activity.

From these observations it is possible to conclude that despite the unusual stability of the active material it is definitely of organic nature. The possibility of the activity being due to a volatile or inorganic compound is excluded.

Precipitations by Heavy Metals

Upon the addition of lead salts to the active colutions a bulky precipitate was obtained which proved to be inactive. The procedure for the preparation of the active solution was then modified to incorporate the use of this reagent at an early stage as follows: the tissue was suspended in water, heated, and filtered as before. A hot saturated solution of lead acetate was slowly added with stirring to the hot tissue extract until no further precipitation took place. After fifteen minutes the solution was filtered and the filtrate was treated with fullers' earth and supercel followed by the charcoal adsorption. The charcoal was freed from lead by washing and the activity was eluted in the manner previously described. By this treatment the solid content of the solutions was decreased by 20-40% without any loss in activity.

Silver, mercury, and platinum salts were still able to precipitate small amounts of the material (1-3%) from the lead-treated solutions. The activity was not precipitated by this procedure although small amounts of activity not removable by washing were occasionally occluded in the precipitates. Furthermore, washing was found to cause the precipitates to pass through the filters as colloids. Both these difficulties were circumvented by sucking the precipitates dry followed by decomposition and reprecipitation. From 5-10% of the solids (but no activity) were lost and the solutions became considerably lighter in color on passing through the hydrogen sulfide precipitation of the metals. It was assumed that some of the inactive material was adsorbed on the metallic sulfides.

The solution at this stage gave negative biuret and ninhydrin tests. By virtue of the treatments (heat coagulation, alcohol extraction, and metallic precipitation) and results of the color tests, the solution can be considered to be protein-free. The more soluble amino acids might, however, have remained and are still to be considered. The solutions were yellow and the usefulness of the color tests were considerably reduced, since slight development of color could be masked by the original yellow color.

Further purification could be effected by the use of metals in alkaline solution. An excess of lead acetate was added to

an active solution and the solution was brought to a pH of 8-9 with dilute ammonium hydroxide. The voluminous precipitate was inactive and represented about 10-15% of the organic solids. Similar results were obtained with silver acetate solutions made alkaline with barium hydroxide. Using these reagents somewhat more inert material could be removed (12-20%). Because of the expense of silver salts and the difficulties in removing the highly toxic barium, this method was abandoned and the lead-ammonia method used instead. After this treatment the solution could be considered to be quite free from carbohydrates.

The Molisch test had been faintly positive before the lead-ammonia treatment and became negative after it. The Fehling's test was negative (both before and after boiling with 5% sulfuric acid for fifteen minutes) on all preparations which had gone through the charcoal treatment. The results of these precipitations offer no evidence for the existence of acid groups. Rather, from the lack of precipitation, it is likely that the active compound is not an acid of any considerable strength.

Solubilities

The active solution on evaporation yielded a non-granular amorphous residue which is not hygroscopic and which dissolves readily and completely in water or phenol. Extracts made with hot methyl, ethyl, isopropyl, and butyl alcohols were found to be effective in the order named in dissolving 40-70% of the residue. These extracts contained most of the active material. Extracts of this residue made using ether, chloroform, carbon tetrachloride or benzene as solvents were inactive. The activity could be precipitated by pouring the methyl or ethyl alcohol extracts into five volumes of absolute ether. By the use of this procedure considerable purification was possible. Using the methyl alcohol-ether procedure, increases of potency of two to three times were effected.

Extraction from the aqueous solution by immiscible liquids is a separatory funnel was attempted but difficulties in obtaining clear results were encountered. Separation of the phases proved to be extremely difficult and activity was found in the non-aqueous phases. Repetition of these experiments using a continuous extraction apparatus showed the activity to be insoluble in the non-

aqueous solutions. The use of an apparatus in which the extracting solvent is passed into the aqueous phase through a sintered glass plate was most satisfactory. The extraction proceeded at a steady rate and the interfacial boundary remained distinct. About 1.0 to 1.5% of the solid content was found to be extractable by ether and this procedure was instituted routinely as a means of removing the small amount of lipoid material from the active solutions.

Phosphotungstic Acid

Fifty cc. of a 25% solution of phosphotungstic acid in 5% sulfuric acid were slowly added to 25 cc. of an active solution containing 1-1.5 gm. of solids. A heavy precipitate formed and the solution became colorless. The solution was filtered and the filtrate was shaken with 20 gm. of solid barium hydroxide for thirty minutes, while the precipitate was shaken for an equal length of time with 150 cc. of saturated barium hydroxide. The filtrates from these procedures were treated to remove the excess toxic ions; barium was removed by sulfuric acid, sulphate by lead acetate, and lead by hydrogen sulfide. The solutions were then evaporated to dryness and the residues were taken up in water and assayed. By careful titration it is possible to eliminate the use of the excess of sulfuric acid and the subsequent lead sulphate and lead sulfide precipitations. This shorter method is not without danger as barium ions are highly toxic to bacteria; hence, the use of an excess of sulfuric acid seemed to be the safer procedure.

From the results recorded in Table 6 it can be seen that most of the activity could be precipitated by phosphotungstic acid, although this precipitation was incomplete. The precipitate was more highly nitrogenous than was the filtrate and showed a concentration of the substances having amino nitrogen. These results point strongly towards the idea that the active substance is an amine. The precipitate was active in concentrations of 1.5 micrograms/cc. Combination of the solutions derived from the precipitate and the filtrate, (C), showed little stimulation of growth, except in the case of Corynebacterium diphtheriae where marked stimulation occurred. It would seem that this organism may have two factors one of which is not precipitated by phosphotungstic

TABLE 6

THE EFFECT OF PRECIPITATION BY PHOSPHOTUNGSTIC ACID

Organism	Amount of Extract Added	(A) Filtrate from "PTA" Treatment	(B) Precipitate from "PTA" Treatment	(C) Equal Volumes of (A) and (B)	(D) Filtrate Prepared as was (A) Showing Poisoning
		in cc. in days 1 2 4	in days 1 2 4	in days 1 2 4	in days 1 2 4 10
Staphylo- coccus albus	0.1	++ +++ +++	++ +++ +++	+++ +++ +++	+ ++ +++ +++
	.01	+ + +	++ ++ ++	++ ++ ++	+ + + ++
	.001		+ ^s + ^s +	+ ^s + ^s +	
	none				
Coryne- bacterium diphtheriae	0.1	.. + +++	.. + ^s +++	+ +++ +++	
	.01	 +		+ ^s + +++	 +++
	.001			 +	 ++
	none				
Shigella dysenteriae	0.1		+ + +	+ ^s + ++	
	.01			 ++	
	.001				
	none				
Brucella abortus	0.1	 +	 ++	 ++	
	.01		 +	 +	
	.001				
	none				
Total Nitrogen Percentage		9.3%	14.7%		
Amino Nitrogen Percentage of Total Nitrogen		23%	42%		

acid. This suggestion has been made by Mueller (12) who found that pimelic acid could act as a stimulant to the growth of Corynebacterium diphtheriae in the presence of another essential factor. While pimelic acid should not be present in our preparation (after lead-precipitation and ether extraction), the possibility of other compounds having this stimulatory property exists and from our results seems likely.

The effects of solution (D) are included in Table 6 as an example of poisoning of the medium. Inhibition of growth is observable with all the organisms except with Staphylococcus albus. Because of the high activity of the growth factor the dilutions reduce the concentration of the toxic substances to a point where they are no longer active. It is this phenomenon of lack of growth followed by growth upon dilution that indicates the presence of toxic substances. When toxic solutions were obtained, the experiments were repeated and, if necessary, the procedures were modified. In the early experiments with phosphotungstic acid toxic solutions were the rule rather than the exception. The use of long and vigorous shaking with barium hydroxide in the removal of the excess phosphotungstic acid and the use of an excess of sulfuric acid in the removal of the barium with subsequent lead sulfate and sulfide precipitations were found to give solutions free from poisons.

Action of Acids and Bases

Two grams (0.050 equivalents) of sodium hydroxide were dissolved in 25 cc. of an active solution and the resultant solution was refluxed for forty-eight hours. The dark solution was then cooled, neutralized and filtered. The filtrate with the washings were evaporated to 25 cc. and 250 cc. of redistilled ethyl alcohol were slowly added to precipitate the salts. The solution was filtered and the filtrate was evaporated to dryness. The residue was taken up in 25 cc. of water and the resulting solution was filtered and assayed. A similar preparation was made using 1.40 cc. (0.052 equivalents) of concentrated sulfuric acid in place of the sodium hydroxide. The properties of these solutions are shown in Table 7.

The effects of these hydrolytic agents on the active solutions were most marked. The results showed a loss of nitrogen

TABLE 7
EFFECTS OF ACIDS AND BASES

Organism	Amount of Extract Added in cc.	(A) Before Treatment	(B) After Basic Treatment	(C) After Acidic Treatment
		in days 1 2 4	in days 1 2 4	in days 1 2 4
Staphylo- coccus albus	0.1	+++ +++ +++	+ ^s +++ +++	+++ +++ +++
	.02	++ +++ +++	... ++ +++	+++ +++ +++
	.004	? + ^s +	+ ++ ++	++ ++ +++
Coryne- bacterium diphtheriae	0.1	+ ++ +++	... + ^s +++	... + ^s +++
	.02	+ ^s ++ +++	... + ^s +++	 ++
	.004	... + ^s ++	 + ^s	
Shigella dysenteriae	0.1	++ ++ +++	+++ +++ +++	+++ +++ +++
	.02		+ + +	++ ++ ++
	.004			+ ^s + ^s + ^s
Brucella abortus	0.1	 ^a	 ++	+++ +++ +++
	.02	 ^b	 + ^s	 +
	.004			
Total Nitrogen Percentage		20.9	10.5	16.1
Amino Nitrogen Percentage of Total Nitrogen		15.2	39.7	37.2

^a++ on 10th day.

^b + on 10th day.

with both reagents (about 25% of the nitrogen was lost by the acid treatment and about 50% by the alkali treatment). These results and the fact that solutions after treatment were almost black show

that extensive destruction had taken place. The differences in the amounts of nitrogen lost in the two cases need not mean that destruction was more extensive in the case of the sodium hydroxide treatment. It may merely mean that whatever nitrogen was hydrolyzed to ammonia or its derivatives was boiled off from the alkaline solution. The fact that the amino nitrogen more than doubled is significant. It proves that, while the solutions may be free from proteins, there are substances present which are not precipitated by metals or alcohol and which can be hydrolyzed to primary amines.

The activity of these solutions was not destroyed by this drastic hydrolytic and oxidative treatment. In fact, we have the curious situation where the growth of Shigella dysenteriae was stimulated to the point of being equal to that usually exhibited by the responsive Corynebacterium diphtheriae. On the other hand, the growth of Corynebacterium diphtheriae was weak and was comparable to that usually obtained with Shigella dysenteriae. The order of response had been definitely inverted! These results are most marked with the acid-treated solution but are also seen in the alkali-treated case. The growth of Staphylococcus albus and Brucella abortus are also stimulated slightly by this treatment. If a growth factor is present in a conjugated form and can be liberated on hydrolysis; and if Corynebacterium diphtheriae requires another factor for full growth which is destroyed by boiling acids or bases, these results become comprehensible.

Effect of Treatment with Nitrous Acid

Several methods of using nitrous acid were employed and the results varied somewhat with the method. In the first method, 10 gm. of sodium nitrite were dissolved in 50 cc. of an active solution and 25 cc. of glacial acetic acid (diluted) were slowly added. The solution was stirred for thirty minutes and then allowed to stand overnight in the refrigerator. The growth factor was adsorbed on 8 gm. of charcoal and eluted with 90% alcohol for one week. When the solutions were assayed, it was found that the activity had decreased somewhat. Experiments were run in which the time, the temperature, and the concentrations of sodium nitrite and acetic acid were varied, but the results always showed a slight decrease in activity. It was thought possible that the

active material was not affected at all by this treatment and the slight decrease in the activity was due to incomplete elution from the charcoal. Other methods were then used in which the charcoal adsorption would be unnecessary.

The second method involved passing the gases from a nitrous acid generator into the solution of the active material. The gases were generated in a 500 cc. Erlenmeyer flask by dropping 200 cc. of 10% sulfuric acid into a solution of 17 gm. of sodium nitrite in 25 cc. of water. The gases were evolved at the rate of 10 cc. per minute and were passed into an empty tube to catch the spray. From this tube they were bubbled into a tube containing 25 cc. of an active solution. When all the gases had been passed in, the solution was evaporated to dryness in vacuo and taken up in 25 cc. of water.

The third method consisted of generating the gases within the active solution itself under pressure. This was the most vigorous form in which the reagent was applied. Six grams of sodium nitrite were dissolved in 25 cc. of growth factor solution in a 250 cc. flask. Fifty cc. of 10% sulfuric acid were added in 2-5 cc. quantities every five to ten minutes and the flask was tightly stoppered. It is estimated that about five to ten pounds extra pressure were developed in this way. The solution was then evaporated to dryness in vacuo, the residue taken up in 25 cc. of water, and the solution neutralized with sodium hydroxide. About 250 cc. of alcohol were slowly added to the neutralized solution and the precipitate of salts filtered off. The alcoholic solution was evaporated to dryness and the residue was taken up in 25 cc. of water.

The results with all three methods were about the same. The activity was not destroyed, although growth in some cases was delayed and weaker than in the controls. The second and third methods were repeated on the same solutions as many as four times with little change in the activity. The results of these treatments (Table 8) show that nitrous acid has a profound effect on the nitrogenous constituents of the solution.

The third method (gas under pressure) is obviously the more effective of the two. The results after a single such treatment are similar to the results after four treatments with the second (bubbling gas) method. With both types of treatment the total nitrogen content drops to a point below that which would

TABLE 8

EFFECT OF NITROUS ACID ON THE NITROGEN CONTENT

	Second Method		Third Method	
	Per cent Nitrogen	Per cent Amino Nitrogen	Per cent Nitrogen	Per cent Amino Nitrogen
Before Treatment	20.9	15.2	20.9	15.2
After 1st Treatment	17.4	12.5	4.9	10.0
After 2nd Treatment	15.4	 ^a	2.3	4.2
After 4th Treatment	10.4	9.4	 ^b	 ^b

^aNot analyzed.

^bNo nitrogen was detectable by either of the quantitative methods nor by a qualitative test after sodium fusion.

be expected from the loss of all the primary amino nitrogen. This indicates that the solution contains relatively large amounts of secondary amines or other substances which react with nitrous acid to give oxidation products other than nitrogen gas. Since the Koch-McMeekin method which does not utilize any reduction process was used, these oxidation products would escape detection. From the relatively slow rate at which the amino nitrogen content decreased, it is likely that the solutions contained primary amines which react more slowly with nitrous acid than do alpha-amino acids. Four successive treatments with nitrous acid using the gas under pressure (third) method produced a preparation which was active but gave no evidence of the presence of nitrogen, even after sodium fusion. This suggests, among other possibilities, that the active principle is non-nitrogenous. However, it is possible that nitrogen is present and yet would escape detection in a sodium fusion.

Miscellaneous Experiments

Dialysis.--Ten cc. of an active preparation were placed in a collodion bag and dialyzed against 25 cc. of distilled water at 3-5°C. The water outside the bag was changed morning and night and the dialysis was run for seven days. (Assuming that equilibrium was attained between the phases with each change of water, 99.9% of the dialyzable material would have been removed from within the dialysis bag after five days.) The dialysate and the colorless solution within the bag were evaporated to dryness and the residues from each were taken up in 10 cc. of water and assayed. More than 99% of the solid material and all of the activity were found to have passed through the dialysis bag. While the results are significant in deciding that the active molecules are relatively small, the method is of little use in fractionation.

Acetylation.--Fifty cc. of an active solution were evaporated to dryness in vacuo. Seventy-five grams of acetic anhydride and 5 gm. of fused sodium acetate were added. The mixture was heated on the steam bath under reflux for twenty minutes and then poured into 500 cc. of ice water. The gummy precipitate formed was filtered off. The filtrate was diluted to two liters and treated with 15 gm. of charcoal in two steps. The charcoal was filtered off and extracted in a continuous extraction apparatus for one week. The alcohol was removed under reduced pressure and the residue was taken up in 50 cc. of water. Assay showed that there had been only a slight loss of activity. No evidence existed that the active compound had acetyltable groups.

Benzoylation.--Two grams of sodium hydroxide were dissolved in 50 cc. of an active solution and 5 gm. of benzoyl chloride were added. The solution was boiled for one minute and the precipitate which formed was filtered off. The filtrate was boiled for thirty minutes, cooled, acidified with sulfuric acid and filtered. The filtrate was extracted with ether and the ether phase was discarded. The aqueous solution was evaporated to 75 cc. and 250 cc. of alcohol were added. The precipitate of salts was filtered off and the solution was evaporated to dryness. The residue was taken up in 50 cc. of water. The precipitate which formed on benzoylation was suspended in 5% sodium hydroxide and boiled for thirty minutes. This solution received the identical treatment for the

removal of benzoic acid that had been given to the filtrate. Assay revealed that the active substance had not formed an insoluble benzoyl derivative. The experiments were repeated using barium hydroxide in place of sodium hydroxide, and m-nitro benzoyl chloride in place of benzoyl chloride. The results were analogous to those obtained with the first reagent used.

Chromium complexes.--Two compounds which have been used successfully for the precipitation of weak amines such as amino acids and peptides are Reinecke's salt, $(\text{NH}_4)[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4]\cdot\text{H}_2\text{O}$, and its aniline homologue, ammonium rhodanilate, $(\text{NH}_4)[\text{Cr}(\text{C}_6\text{H}_5\text{NH}_2)_2(\text{SCN})_4]\cdot 1\frac{1}{2}\text{H}_2\text{O}$. Reinecke's salt was prepared and used according to the methods given by Dakin (3, 4). For the preparation and use of the rhodanilate, Bergmann's procedures were followed (1). The solutions derived from the precipitates with the chromium complexes were not active, while the filtrates retained their original activity and 68-73% of their original organic solid contents.

SUMMARY

1. Starting with an active charcoal elutate (0.5-5.0 micrograms/cc.), considerable purification could be effected by (a) precipitation of inert material with lead in neutral and in alkaline solution, (b) extraction with ether, (c) extraction of the dried residue with methyl alcohol, and (d) precipitation of the active material from the methyl alcohol extract with ether. In this way a preparation could be obtained which was free from carbohydrates, fats, and proteins; and this was active in a concentration of 0.08-0.15 micrograms/cc.

2. The effective agent (or agents) has been shown to be a non-volatile, dialyzable, thermostable, difficultly-oxidizable organic substance.

3. While the activity is readily soluble in water, no evidence for the existence of polar hydroxyl and amino groups was obtained from benzoylation or acetylation. No evidence for the existence of acidic groups was obtained from metallic precipitations.

4. An active nitrogen-free preparation was obtained by nitrous acid treatment. This conflicts with the evidence obtained from phosphotungstic acid precipitation which indicated that the

activity was associated with the nitrogenous bases. The question of the importance of nitrogen must remain open for the present.

5. Hydrolytic agents, for the most part, enhance rather than diminish growth. The presence of conjugated amines in the active solutions can be inferred from the large increase of alpha-amino nitrogen on hydrolysis.

6. Inversions of the "order of response" of the various bacteria to active solutions have been observed (a) with elutions under various conditions and (b) by hydrolysis with acids and bases.

7. Evidence for the existence of more than one factor for Corynebacterium diphtheriae was obtained. Growth with the combined solutions resulting from the phosphotungstic acid precipitate and the filtrate was greater than with the solution derived from either the precipitate or the filtrate. Growth with hydrolyzed solutions was markedly retarded and weak indicating the destruction of a rather specific non-essential growth stimulant.

LIST OF REFERENCES

1. Bergmann, M., Amino acids and peptides: II. J. Biol. Chem. 110: 476. 1935.
2. Burrows, W., The nutritional requirements of bacteria. Quart. Rev. Biol. 11: 406. 1935.
3. Dakin, H. D., Reinecke's Salt. Vol. XV, Organic Syntheses. New York: John Wiley & Sons, 1935. P. 74.
4. Dakin, H. D., Observations on the chemical nature of the hematopoietic substance in liver. J. Biol. Chem. 109: 489. 1935.
5. Hughes, T. P., Growth requirements of Staphylococcus. J. Bact. 23: 437. 1932.
6. Knight, B. C. J. G., The essential growth factor for Staphylococcus aureus. Brit. J. Exp. Path. 16: 315. 1935.
7. Knight, B. C. J. G., Bacterial Nutrition. Medical Research Council Special Report No. 210. London: H. M. Stationery Office, 1936.
8. Koch, F. C., Practical Methods of Biochemistry. Baltimore: Wm. Wood & Co., 1934. P. 182.
9. Koser, S. A., and Saunders, F., Studies on bacterial nutrition: I. Separation of the growth factors from veal infusion. J. Infectious Diseases 56: 305. 1935.
10. Koser, S. A. and others, Studies on bacterial nutrition: II. The distribution of a growth stimulating factor in plant and animal tissues. J. Infectious Diseases 58: 121. 1936.
11. Mueller, J. H., Studies on the cultural requirements of bacteria: II. J. Bact. 7: 325. 1922.
12. Mueller, J. H., Pimelic acid as a growth accessory for the diphtheria bacillus. J. Biol. Chem. 119: 121. 1937.
13. Peskett, G. L., Growth factors of lower organisms. Biol. Rev. 8: 1. 1933.
14. Pappenheimer, Jr., A.M., The nature of the Sporogenes vitamin. Biochem. J. 29: 2056. 1935.
15. Richardson, G. M., Nutrition of Staphylococcus aureus. Necessity of uracil in anaerobic growth. Biochem. J. 30: 2184. 1936.
16. Sahyan, M. and others, Growth stimulating factors for micro-organisms. J. Infectious Diseases 58: 28. 1936.

17. Saunders, F. and others, Some chemical properties of an essential growth factor for pathogenic bacteria. J. Am. Chem. Soc. 59: 170. 1937.
18. Van Slyke, D. D., Note on a portable form of the manometric gas apparatus. J. Biol. Chem. 73: 121. 1927.

